

In the specification

On page 1, after the title of the application and before "Field of the Invention", please add the following paragraph:

This application is a continuation of and claims priority to pending prior application U.S.S.N. 09/694,836 filed October 23, 2000 by Jeffrey A. Hubbell, Chandrashekhar P. Pathak, Amarpreet S. Sawhney, Neil P. Desai, Jennifer L. Hill, and Syed F. A. Hossainy entitled "Gels for Encapsulation of Biological Materials", which is a continuation of U.S.S.N. 09/033,871 filed March 3, 1998, which is a continuation of U.S.S.N. 08/467,693 filed June 6, 1995, now U.S. Patent No. 5,834,274, which is a divisional of U.S.S.N. 08/024,657 filed March 1, 1993, now U.S. Patent No. 5,573,934, which is a continuation-in-part of U.S.S.N. 07/958,870, filed October 7, 1992, now U.S. Patent No. 5,529,914, which is a continuation-in-part of U.S.S.N. 07/870,540, filed April 20, 1992 (now abandoned).

Please replace the paragraphs on page 7, lines 4 through 10, with the following paragraph:

Figure 2A 2 is a schematic of dye-initiated polymerization of a PEG layer around crosslinked alginate microspheres.

~~Figure 2B is a photomicrograph of the alginate/poly(L-lysine) microspheres containing human islets of Langerhans coated with a PEG 18.5K tetraacrylate hydrogel using the dye binding method depicted in Figure 2A.~~

Please replace the paragraphs on page 7, lines 14 through 32, with the following paragraphs:

~~Figure 4 is a photomicrograph of Islets of Langerhans isolated from a human pancreas encapsulated in a PEG 18.5K tetraacrylate hydrogel.~~

Figure ~~5~~ 4 is a schematic representation of coextrusion apparatus used for microencapsulation using laser polymerization.

~~Figure 6 is a photomicrograph of microspheres produced by laser polymerization of PEG 40 diacrylate around cells.~~

~~Figure 7A is a photomicrograph of alginate-PLL microspheres recovered after 4 days following implantation i.p. in mice.~~

~~Figure 7B is a photomicrograph of Alginate-PLL microspheres coated with a PEG 18.5K Da tetraacrylate, using the dye diffusion method depicted in Figure 1.~~

~~Figures 8A-F~~ Figure 5 is a graph of the number of cells versus gel composition, for the unattached cells obtained from lavage of the peritoneal cavity in mice with different PEO overcoat gel compositions: a – 18.5k; b – 10% 0.5k, 90% 18.5k; c – 50% 18.5k, 50% 0.4k; d - 10% 0.4k, 90% 35k; e – 50% 0.4k, 50% 35k; and f – alginate-poly(L-lysine) control.

Please replace the paragraphs on page 8, lines 1 through 27, with the following paragraphs:

Figure 9 ~~6~~ is a graph of the % protein released versus time in minutes, for diffusion of bovine serum albumin (open squares), human IgG (triangles) and human fibrinogen (closed squares) through a PEO 18.5K-tetraacrylate gel.

Figure 10 ~~7~~ is a graph of the % diffusion of bovine serum albumin over time in minutes through PEO 400 diacrylate (open squares) and PEG 18.5K-tetraacrylate (triangles) gels.

Figure 11A ~~8~~ is a graph of the length in mm of gel produced by argon ion laser induced polymerization versus log (time) (ms) of trimethylolpropane using an amine and ethyl eosin initiation system.

~~Figure 11B is a photomicrograph of the spikes formed as a result of laser irradiation of ethoxylated trimethylol propane triacrylate for durations of 67 ms, 125 ms, 250 ms, 500 ms, and 1 sec.~~

~~Figure 12A is a photomicrograph of human foreskin fibroblasts cultured for 6 h on a glass coverslip coated with PEG 18.5K-tetraacrylate gel.~~

~~Figure 12B is a photomicrograph of human foreskin fibroblasts cultured for 6 h on a glass that was not coated with PEG.~~

~~Figure 13 is a photomicrograph of PEG 18.5K-tetraacrylate microspherical gels, implanted in mice, and explanted after 4 days, showing very little fibrous overgrowth.~~

Figures ~~14A-C~~ 9A, 9B and 9C are creep curves for PEG diacrylate and tetraacrylate gels; test and recovery loads are given below the abscissa: A – 1k; B – 6K; and C – 18.5K PEG gels.

Please delete the paragraph on page 31, lines 28 through 29, as follows:

~~Alginate/PLL microspheres containing islets coated by this technique are shown in Figure 2B.~~

Please replace the paragraph on page 34, lines 1 through 14, with the following paragraph:

~~Figure 4 shows islets of Langerhans encapsulated in a PEG-tetraacrylate gel.~~ The viability of ~~the Islets of Langerhans encapsulated in a PEG-tetraacrylate gel~~ islets of Langerhans encapsulated in a PEG-tetraacrylate gel was verified by an acridine orange and propidium iodide staining method and also by dithizone staining. In order to test functional normalcy, a SGS test was performed on these islets. The response of the encapsulated islets was compared to that of free islets maintained in culture for the same time period. All islets were maintained in culture for a week before the SGS was performed. The results are summarized in Table 2. It can be seen that the encapsulated islets secreted significantly ($p < 0.05$) higher amounts of insulin than the free islets. The PEG-tetraacrylate gel encapsulation process did not impair function of the islets and in fact helped them maintain their function in culture better than if they had not been encapsulated.

Please replace the paragraph bridging pages 34 and 35 with the following paragraph:

PEG diacrylates of different molecular weight were synthesized by a reaction of acryloyl chloride with PEG as in Example 1. A 20 to 30% solution of macromer was mixed with a cell

suspension and the ethyl eosin and triethanolamine initiating system before exposing it to laser light through a coextrusion air flow apparatus shown in Figure 5 4. Microspheres were prepared by an air atomization process in which a stream of macromer was atomized by an annular stream of air. The air flow rate used was 1,600 cc/min and macromer flow rate was 0.5 ml/min. The droplets were allowed to fall into a petri dish containing mineral oil and were exposed to laser light for about 0.15 sec each to polymerize the microspheres and make them insoluble in water. The microspheres were separated from the oil and thoroughly washed with PBS buffer to remove unreacted macromer and residual initiator. The size and shape of the microspheres was dependent on extrusion rate (0.05 to 0.1 ml/min) and extruding capillary diameter (18 Ga to 25 Ga). The polymerization times were dependent on initiator concentration (ethyl eosin concentration (5 μ M to 0.5 mM), vinyl pyrrolidone concentration (0.0% to 0.1%), triethanolamine concentration (5 to 100 mM), laser power (10 mW to 1 W), and macromer concentration (greater than 10% w/v).

Please replace the paragraph on page 35, lines 13 through 21, with the following paragraph:

A PEG diacrylate macromer of molecular weight 400 Da was used as a 30% solution in PBS, containing 0.1 M triethanolamine as a cocatalyst and 0.5 mM ethyl eosin as a photoinitiator. ~~Spheres prepared using this method are shown in Figure 6.~~ The polymerizations were carried out at physiological pH in the presence of air. This is significant since radical

polymerizations may be affected by the presence of oxygen, and the acrylate polymerization is still rapid enough to proceed effectively.

Please replace the paragraph on page 38, lines 6 through 21, with the following paragraph:

~~Figure 7A shows a photograph~~ Photographs were taken of alginate-poly(L-lysine) microspheres recovered after 4 days, ~~while Figure 7B shows and~~ similar spheres which had been coated with PEG gel by the dye diffusion process before implantation. As expected, bilayer alginate-polylysine capsules not containing an outer alginate layer were completely covered with cells due to the highly cell adhesive nature of the PLL surface, whereas the PEG coated microspheres were virtually free of adherent cells. Almost complete coverage of alginate-poly(L-lysine) was expected because polylysine has amino groups on the surface, and positively charged surface amines can interact with cell surface proteoglycans and support cell growth (Reuveny, *et al.*, (1983) *Biotechnol. Bioeng.*, 25:469-480). The photographs ~~in Figure 7B~~ strongly indicate that the highly charged and cell adhesive surface of PLL is covered by a stable layer of PEG gel. The integrity of the gel did not appear to be compromised.

Please replace the paragraph on page 39, lines 10 through 19, with the following paragraph:

An increase in cell count is a result of activation of resident macrophages which secrete chemical factors such as interleukins and induce nonresident macrophages to migrate to the implant site. The factors also attract fibroblasts responsible for collagen synthesis. The variation

of cell counts with chemical composition of the overcoat is shown in Figure 8 (A-F) 5 (a-f). It can be seen from the figure that all PEG coated spheres have substantially reduced cell counts. This is consistent with the PEG overcoat generally causing no irritation of the peritoneal cavity.

Please replace the paragraph bridging pages 39 and 40 with the following paragraph:

20 mg of bovine serum albumin, human IgG, or human fibrinogen was dissolved in 2 ml of a 23% w/v solution of oligomeric PEG 18.5k tetraacrylate in PBS. This solution was laser polymerized to produce a gel 2 cm X 2 cm X 0.5 cm in size. The diffusion of bovine serum albumin, human IgG and human fibrinogen (mol wt 66 kDa, 150 kDa and 350 kDa respectively) was monitored through the 2 cm X 2 cm face of these gels using a total protein assay reagent (Biorad). A typical release profile for PEG 18.5K gel was shown in Figure 9 6. This gel allowed a slow transport of albumin but did not allow IgG and fibrinogen to diffuse. This indicates that these gels are capable of being used as immunoprotective barriers. This is a vital requirement for a successful animal tissue microencapsulation material.

Please replace the paragraph on page 40, lines 6 through 18, with the following paragraph:

The release profile was found to be a function of crosslink density and molecular weight of the polyethylene glycol segment of the monomer. Figure ~~10~~ 7 shows the release of bovine serum albumin (BSA) through gels made from 23% solutions of PEO diacrylates and tetraacrylates of 0.4K and 18.5K, respectively. It is evident that the 18.5K gel was freely permeable to albumin while the 0.4K gel restricted the diffusion of albumin. The release of any

substance from these gels depends on the crosslink density of the network and also depends on the motility of the PEG segments in the network. This effect was also dependent upon the functionality of the macromer. For example, the permeability of a PEG 18.5K tetraacrylate gel was less than that of an otherwise similar PEG 20K diacrylate gel.

Please replace the paragraph on page 41, lines 9 through 21, with the following paragraph:

The kinetics of a typical reaction were determined to demonstrate rapidity of gelation in laser-initiated polymerizations of multifunctional acrylic monomers. Trimethylolpropyl triacrylate, containing 5×10^{-4} M ethyl eosin as a photoinitiator in 10 μ moles of N-vinyl pyrrolidone per ml of macromer mix and 0.1 M of triethanolamine as a cocatalyst, was irradiated with a 500 mW argon ion laser (514 nm wavelength, power 3.05×10^5 W/m², beam diameter 1 mm, average gel diameter produced 1 mm). A plot of the length of the spike of gel formed by penetration of the laser beam into the gel versus laser irradiation time is shown in Figure 11A. 8. ~~The spikes formed as a result of laser light penetration into the macromer can be seen in Figure 11B.~~

Please replace the paragraphs on page 43, lines 3 through 17, with the following paragraphs:

~~Figure 12A shows the~~ The growth of these cells on a typical PEG gel as was compared to the growth of these cells on a glass surface (Figure 12B). The number of attached cells/cm² was found to be 510 ± 170 on the gel surfaces as compared to $13,200 \pm 3,910$ for a control glass

surface. The cells on these gels appeared rounded and were not in their normal spread morphology, strongly indicating that these gels do not encourage cell attachment.

Biocompatibility on microspheres was demonstrated as follows. ~~Figure 13 shows a~~ A photograph of microspheres explanted from mice as in Example 10 was taken; after 4 days very little fibrous overgrowth is seen. The resistance of PEG chains to protein adsorption and hence cellular growth is well documented. Table 5 summarizes the extent of cellular overgrowth seen on these microspheres formed of various PEG diacrylate gels after implanted intraperitoneally for four days.

Please replace the paragraph on page 45, lines 1 through 15, with the following paragraph:

For the creep tests, eight samples approximately 0.2 X 0.4 X 2 cm were loaded while submersed in saline solution. They were tested with a constant unique predetermined load for one hour and a small recovery load for ten minutes. Gels made from PEG diacrylates of 1K, 6K, and 10K, and PEG tetraacrylates of 18.5K PEG molecular weight were used for this study. The 10K test was terminated due to a limit error (the sample stretched beyond the travel of the loading frame). The 1K sample was tested with a load of 10 g and a recovery load of 0.2 g. The 6K sample was tested at a load of 13g with a recovery load of 0.5g. The 18.5K sample was tested at a load of 13g with a recovery load of 0.2g. The choice of loads for these samples produced classical creep curves with primary and secondary regions. The traces for creep for the 1K, 6K, and 18.5K samples appear in ~~Figure 14A-C~~ Figures 9A, 9B, and 9C, respectively.